

# Implication of $K_v7$ Channels in the Spinal Antinociceptive Actions of Celecoxib

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## ABSTRACT

Celecoxib is a nonsteroidal anti-inflammatory drug (NSAID) commonly used to treat pain conditions in humans. In addition to its blocking activity on cyclooxygenase (COX) enzymes, several other targets could contribute to its analgesic activity. Here we explore the spinal antinociceptive actions of celecoxib and the potential implication of  $K_v7$  channels in mediating its effects. Spinal cord *in vitro* preparations from hind paw-inflamed animals were used to assess the segmental sensory-motor and the early sensory processing of nociceptive information. Electrophysiological recordings of ventral roots and dorsal horn neurones were obtained, and the effects of celecoxib and  $K_v7$  modulators on responses to repetitive dorsal root stimulation at C-fiber intensity were assessed. Celecoxib applied at clinically relevant concentrations produced depressant effects on responses to

dorsal root stimulation recorded from both ventral roots and individual dorsal horn neurones; by contrast, the non-nociceptive monosynaptic reflex was unaffected. The NSAID indomethacin had no effect on spinal reflexes, but further coapplication of celecoxib still produced depressant effects. The depressant actions of celecoxib were abolished after  $K_v7$  channel blockade and mimicked by its structural analog dimethyl-celecoxib, which lacks COX-blocking activity. The present results identify  $K_v7$  channels as novel central targets for celecoxib, which may be relevant to its analgesic effect. This finding contributes to better understand the pharmacology of celecoxib and reinforces both the role of  $K_v7$  channels in modulating the excitability of central pain pathways and its validity as target for the design of analgesics.

## Introduction

Celecoxib, a nonsteroidal anti-inflammatory drug (NSAID) with selective activity on cyclooxygenase 2 (COX-2) isozymes, is used for the treatment of rheumatoid arthritis and osteoarthritis in humans (Clemett and Goa, 2000). Celecoxib applied systemically is effective in reducing nociceptive behaviors in various animal models of pain, including the formalin test and the carrageenan model of paw inflammation (Penning et al., 1997; Zhang et al., 1997; Smith et al., 1998; Inoue et al., 2009; Sun et al., 2013). In addition, the application of celecoxib and other NSAIDs by the intrathecal route also produces antinociceptive effects in the same models (MalMBERG and Yaksh, 1992; Dirig et al., 1998; Nishiyama, 2006; Lee and Seo, 2008). These reports suggest that, in addition to peripheral actions, the spinal cord may be a relevant site for NSAID-induced analgesia. In addition to its COX-blocking actions, celecoxib has been reported to interact with opioid and cannabinoid systems as well as a variety of ionic channels (Park et al., 2007; Brueggemann et al., 2009; Du et al., 2011; Rezende et al., 2012; Frolov and Singh, 2014).

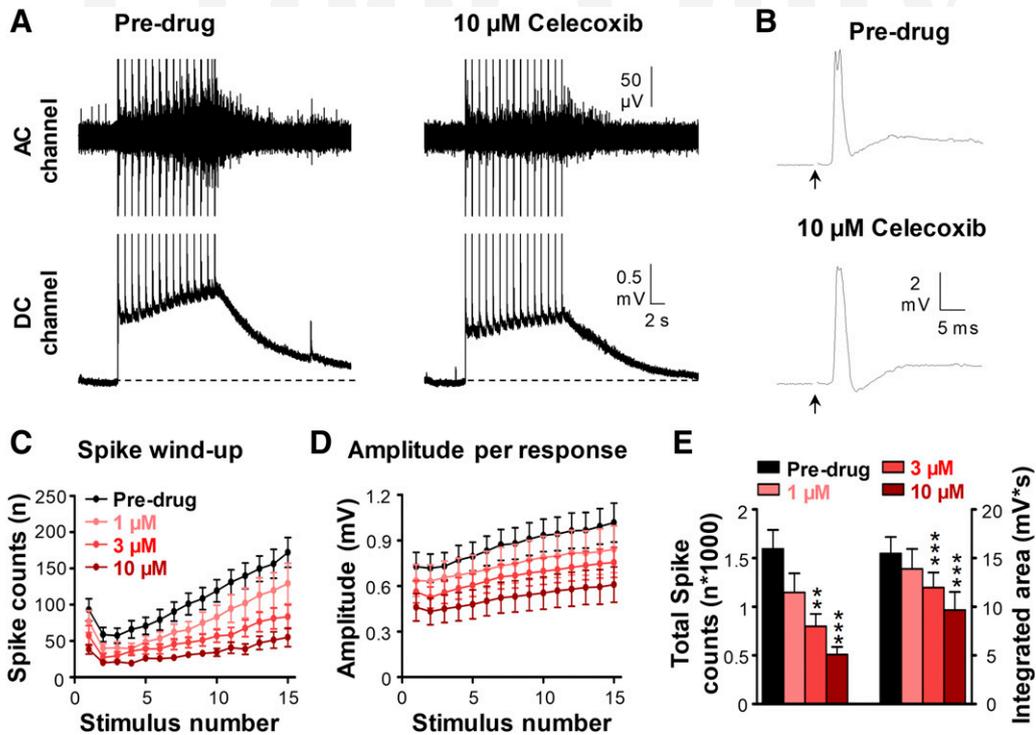
Of special interest for nociceptive processing, celecoxib affects the activity of voltage-dependent potassium channels of the  $K_v7$  family (Du et al., 2011). These channels are expressed in peripheral and central elements of the nociceptive system (Rivera-Arconada et al., 2009), and their modulation alters the excitability of primary afferents and spinal neurones due to its fundamental role regulating membrane resting potential and repetitive firing (Jentsch, 2000; Passmore et al., 2003; Rivera-Arconada and Lopez-Garcia, 2005). The  $K_v7$  channel opener retigabine when applied systemically shows analgesic-like effects on animal models of inflammatory, visceral, neuropathic, and cancer pain (Blackburn-Munro and Jensen, 2003; Passmore et al., 2003; Hirano et al., 2007; Zheng et al., 2013). Application of celecoxib to dorsal root ganglion neurones *in vitro* produces an enhancement of  $K_v7$ -mediated currents, an effect that may be relevant to its analgesic actions (Mi et al., 2013).

Here we explore the potential contribution of  $K_v7$  channels, as expressed in spinal neurones, to the analgesic effects of celecoxib. To this end, we used electrophysiologic recordings of spinal reflexes and dorsal horn neurones in an *in vitro* preparation of the mouse spinal cord obtained after the induction of inflammation. The effect of celecoxib on spinal function was assessed and the contribution of  $K_v7$  channels was tested using a variety of pharmacologic tools. Our results are

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**ABBREVIATIONS:** aCSF, artificial cerebrospinal fluid; COX, cyclooxygenase; Dil, (2Z)-2-[(E)-3-(3,3-dimethyl-1-octadecylindol-1-ium-2-yl)prop-2-enylidene]-3,3-dimethyl-1-octadecylindole; perchlorate; DMC, 2,5-dimethyl-celecoxib; ICA-069673, *N*-(2-chloro-5-pyrimidinyl)-3,4-difluorobenzamide; MEA, multielectrode array; ML 213, *N*-(2,4,6-trimethylphenyl)-bicyclo[2.2.1]heptane-2-carboxamide; MSR, monosynaptic reflex; NSAID, nonsteroidal anti-inflammatory drug; XE-991, 10,10-bis(pyridin-4-ylmethyl)anthracen-9-one.



**Fig. 1.** Actions of celecoxib on ventral root reflexes. This figure summarizes celecoxib's effects on ventral root responses elicited by repetitive stimulation of the dorsal root at C-fiber intensity as obtained in spinal cords from inflamed mice. (A) Original recordings showing the responses in control conditions (left) and after application of 10  $\mu\text{M}$  celecoxib (right). In control conditions, repetitive stimulation (large vertical lines) originates a cumulative depolarization in the DC channel associated with spike wind-up in the AC channel (gradual thickening of baseline). Note the depressant effects of celecoxib on both parameters. (B) Original recordings showing the MSR before and after application of celecoxib. Note the lack of effect of celecoxib on MSR amplitude. Arrows indicate the stimulus artifacts, which have been eliminated to improve visualization. In A and B, three increasing concentrations were tested but only the maximal concentration used is shown. (C and D) A quantification of spike counts (C) and amplitude (D) as obtained for each successive stimulus of the train ( $n = 10$ ). Celecoxib produced a concentration-dependent reduction in spike wind-up and cumulative depolarization. (E) Graph summarizing the effects of the three concentrations of celecoxib tested on the total spike counts ( $F[3,27] = 16.02$ ;  $P < 0.001$ ; one-way ANOVA) and the integrated area of the cumulative depolarization ( $F[3,27] = 18.45$ ;  $P < 0.001$ ; one-way ANOVA). Asterisks indicate significant differences from predrug values using Bonferroni post hoc test (\*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ).

in agreement with the hypothesis that modulation of  $K_v7$  channels is relevant to the spinal antinociceptive actions of celecoxib.

## Materials and Methods

All experimental protocols were performed following the European Union and Spanish Government regulations for animal handling, were approved by the local ethics committee and the Government of the Community of Madrid (Ref. PROEX 018/16) and comply with the ARRIVE guidelines. Experiments were performed on 6- to 11-day-old CD1 mice of either sex weighing between 4 and 9.7 g. The animals were bred in house, kept together with their mothers, and were maintained under a 12-hour light/dark cycle with  $55\% \pm 15\%$  humidity. The number of animals used was kept to the minimum that allowed a clear interpretation of main results. All efforts were made to minimize animal suffering.

**Induction of Peripheral Inflammation and Behavioral Testing.** Peripheral inflammation was induced by intraplantar injection of carrageenan (3% in saline; 30  $\mu\text{l}$ ) in both hind paws. This procedure ensured maximum inflammation and allowed the use of either side of the cord in electrophysiologic experiments. Paw diameters and mechanical withdrawal thresholds were measured before and 20 hours after carrageenan injection. The withdrawal threshold was defined as the minimum mechanical force applied with von Frey filaments (from 0.008 to 2 g) that elicited a withdrawal response in at least three out of five trials. For statistical analyses, the threshold and paw diameter of the hind paw

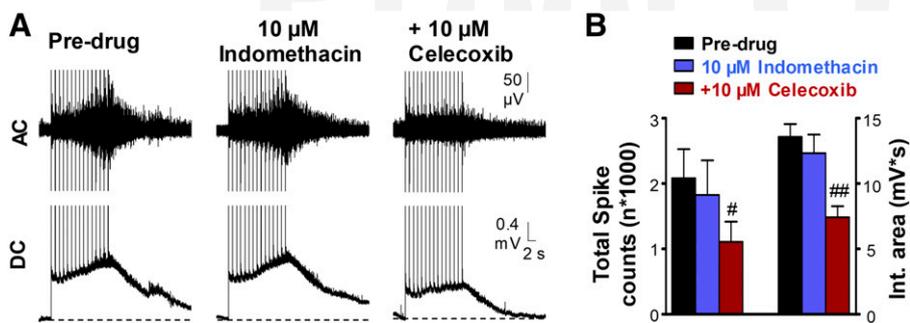
ipsilateral to the hemi cord used for electrophysiologic recordings were considered.

**In Vitro Spinal Cord Preparations and Dorsal Root Stimulation.** Mice were anesthetized with urethane (2 mg  $\text{kg}^{-1}$ , i.p.), and their spinal cords were extracted after a dorsal laminectomy following a procedure described in full elsewhere (Lopez-Garcia and Laird, 1998). The whole cord was used for recording spinal reflexes. The spinal cord was hemisected to make multielectrode recordings from deep dorsal horn neurones. To record neurones located in superficial laminae, the lumbar enlargement of the whole spinal cord was sliced in a vibratome to obtain a single slice of  $\approx 400 \mu\text{m}$  containing the dorsal part of the cord (laminae from I to V) together with the attached dorsal roots.

In the recording chamber, the cord was pinned down to the Sylgard base of the chamber with the medial or sectioned side upward to facilitate electrode penetration. Cords were continuously superfused (4–6 ml  $\text{min}^{-1}$ ) with oxygenated (95%  $\text{O}_2$ ; 5%  $\text{CO}_2$ ) artificial cerebrospinal fluid (aCSF) at room temperature ( $22 \pm 1^\circ \text{C}$ ). The composition of the aCSF was (in millimolars): NaCl (127), KCl (1.9),  $\text{KH}_2\text{PO}_4$  (1.5),  $\text{MgSO}_4$  (1.3),  $\text{CaCl}_2$  (2),  $\text{NaHCO}_3$  (22), and glucose (10); pH 7.4.

Electrical stimuli were applied to the L4 dorsal root via tight-fitting glass suction electrodes. Electrical stimulation consisted of a train of 15 consecutive C-fiber intensity stimuli (200  $\mu\text{A}$ , 200 microseconds) applied at 1 Hz to produce wind-up responses. Trains of stimuli were applied at 30-minute intervals.

**Ventral Root Reflex Recordings.** The L4 ventral root was placed in a tight-fitting glass suction electrode. Signals were obtained using a Multiclamp 7A amplifier (Molecular Devices,



**Fig. 2.** Celecoxib still depressed spinal reflexes after indomethacin application. (A) Original recordings showing the lack of effect of indomethacin ( $P = 0.16$  for cumulative depolarization, and  $P = 0.33$  for spike wind-up; paired  $t$  test) and the subsequent actions of celecoxib. In the presence of indomethacin, celecoxib still produces a profound depression of nociceptive reflexes ( $P = 0.01$ ;  $P = 0.04$  for cumulative depolarization and spike wind-up, respectively; paired  $t$  test). (B) Graph summarizing the results obtained in the six experiments made. # $P < 0.05$ ; ## $P < 0.01$  stand for statistically significant differences between indomethacin and celecoxib values as obtained with paired  $t$  test.

Sunnyvale, CA), were sampled at 6 kHz, and were stored for analysis using Spike 2 software (CED, Cambridge, United Kingdom). Depolarization of motoneuronal populations was recorded in DC channel.

Repetitive dorsal root stimulation at C-fiber intensity produced a cumulative depolarization. For each of the 15 stimuli, the amplitude from baseline was measured at the end of the response. The integrated area under the curve during the 24 seconds after the first stimulus artifact was measured to quantify the cumulative depolarization. Electrical stimuli elicited a monosynaptic reflex (MSR) at 5–10 milliseconds from stimulus artifact. The MSR was quantified as amplitude from baseline using the first stimulus of the train.

Digital band-pass filtering of the original DC signal between 200 and 1200 kHz allowed us to generate an AC channel to collect fast spike-like events produced by the firing of action potentials in motor neurones (Rivera-Arconada et al., 2016). Events crossing an amplitude threshold were counted in a time window between 100 and 950 milliseconds from stimulus artifact and summed to obtain the total spike counts to each train of stimuli. Stable baseline responses were usually obtained after two to four repetitions.

**Multielectrode Recordings from Dorsal Horn Neurones and Estimation of Recording Sites.** Multielectrode arrays (MEAs; A4 × 4 3 mm-50/100-125-177-A16 or Buzsaki32; NeuroNexus Technologies, Ann Arbor, MI) had four shanks with four to eight iridium recording sites separated by 30–100 μm. The MEA tips were placed under visual guidance and were introduced using a remote-controlled micromanipulator. For recordings of deep dorsal horn neurones, extracellular signals were preamplified ×10 (MPA81; Multichannel Systems, Reutlingen, Germany) and were further amplified ×500 with an 8-channel amplifier (Cyberamp; Molecular Devices) (Roza et al., 2016). In recordings from superficial dorsal horn neurones, the electrode was connected to a RHS2000 32-channel headstage containing two RHS2116 amplifier chips (Intan Technologies, Los Angeles, CA) (Lucas-Romero et al., 2018). The signals were band-pass filtered between 200–300 Hz and 3 kHz, digitized at 20 kHz (1401 Plus; CED), and stored for analysis.

Spike sorting was performed offline based on spike-shape parameters using Spike 2. Only data obtained from unequivocally classified units were considered as single unit activity. One to five neurones were isolated from each experiment. Action potentials elicited by dorsal root stimulation were counted excluding the 50 milliseconds after stimulus artifact. Drugs were applied only if responses to repetitive stimuli were stable during two or three stimulation cycles. Responses of dorsal horn neurones recorded with MEAs were reproducible during long-lasting (1.5-hour) applications of the solvent DMSO (12 neurones from three different preparations).

To estimate the position of the deep dorsal horn neurones recorded, the electrode position inside the cord was estimated by DiI labeling [(2Z)-2-[(E)-3-(3,3-dimethyl-1-octadecylindol-1-ium-2-yl)prop-2-enylidene]-3,3-dimethyl-1-octadecylindole; perchlorate (Sigma-Aldrich, Madrid, Spain)]. At the end of a successful track, a microelectrode with identical characteristics stained with DiI was placed in the micromanipulator and lowered to the recording position. This electrode substitution was performed to avoid unnecessary exposure of neurones to

DiI and to prevent damage to the recording electrode. Then the spinal cord was fixed by immersion in 4% paraformaldehyde, embedded in gelatin, and sectioned in a cryostat at 100 μm.

DiI fluorescence was visualized directly onto sectioned tissue, and the depth and trajectory of the electrode were recovered (Roza et al., 2016). After the recording session, the slice was fixed in 4% paraformaldehyde, embedded in gelatin, and sectioned in a cryostat at 30 μm. Then sections were stained with toluidine 0.1% and dehydrated to obtain an estimation of the laminae contained in the slice. In recordings from superficial dorsal horn neurones, the distance of the electrode tip to the dorsal border of the spinal cord was measured with the micromanipulator.

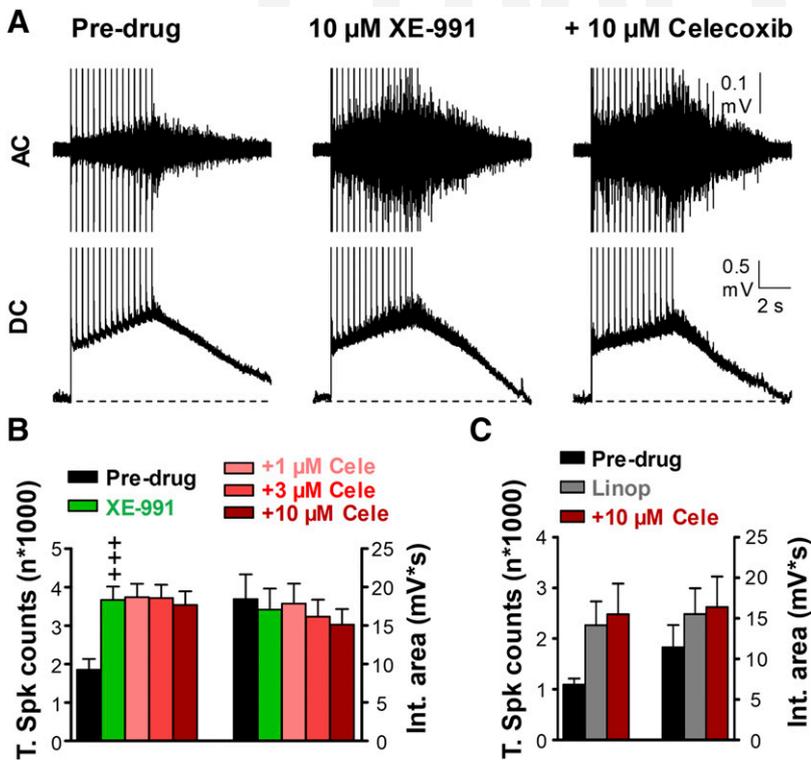
**Drugs and Chemicals.** Carrageenan lambda, celecoxib, 10,10-bis(pyridin-4-ylmethyl)anthracen-9-one (XE-991), indomethacin, 2,5-dimethyl-celecoxib (DMC), and the components for the aCSF were purchased from Sigma-Aldrich. Linopirdine was acquired from Tocris Bioscience (Bristol, United Kingdom). The aCSF was freshly prepared for each experiment. The compounds were prepared in DMSO as concentrated stock solutions (10–20 mM) and stored at  $-20^{\circ}\text{C}$ . All compounds were diluted down to the final concentration in aCSF just before use and were superfused to the entire preparation for at least 25-minute periods to ensure a complete tissue equilibration.

To study the effect of the compounds, each preparation received applications of only one of the compounds at a maximum of three increasing concentrations. In experiments designed to assess the implication of COX enzymes, 10 μM indomethacin was applied during 60 minutes and then 10 μM celecoxib was added to the perfusion medium. In other series of experiments, the  $K_{v}7$  channel blockers XE-991 or linopirdine were superfused for 60–90 minutes before the application of celecoxib or DMC.

**Statistical Analysis.** Statistical analysis was performed with GraphPad Prism (GraphPad Software, San Diego, CA). All data are presented as mean ± S.E.M. unless otherwise stated. Analyses of data from behavioral assays were made using Wilcoxon matched pairs test. The effects of celecoxib on spinal reflexes were first compared with control experiments with DMSO applications using the same three concentrations schedule to avoid a misinterpretation of drug actions due to the long-lasting protocol employed. These comparisons were done using a two-way ANOVA test with data normalized to predrug (or presolvent) values. Concentration-dependent effects of the drugs from predrug values were analyzed in raw data using Bonferroni post hoc test after repeated measures one-way ANOVA. For electrophysiologic studies with a single concentration of drug, the comparisons to predrug values were performed using paired  $t$  test analysis.

## Results

**Celecoxib Actions on Spinal Reflexes Recorded from Paw-Inflamed Mice.** Intraplantar injections of carrageenan induced an inflammatory process in mice pups characterized by paw swelling and allodynia. Twenty hours after carrageenan injection, paw diameter increased from  $2.18 \pm 0.02$  to



**Fig. 3.** Implication of  $K_v7$  channel modulation in the effects of celecoxib. (A) Original recordings showing the lack of effect of celecoxib after  $K_v7$  channel blockade with  $10 \mu\text{M}$  XE-991. XE-991 applied during 90 minutes produced an increase in spike firing; then celecoxib (Cele), coapplied at three increasing concentrations, was devoid of effect. (B) Graph summarizing results obtained in seven experiments. XE-991 increased firing ( $P < 0.001$ ; paired  $t$  test) with no effect on the integrated area of the cumulative depolarization ( $P = 0.082$ ; paired  $t$  test). In the presence of XE-991 no effects of celecoxib were observed ( $F[3,18] = 2.0$ ;  $P = 0.15$ , for cumulative depolarization and  $F[3,18] = 0.3$ ;  $P = 0.8$ , for spike wind-up; one-way ANOVA). (C) Graph showing the results obtained in the four experiments in which celecoxib ( $10 \mu\text{M}$ ) was applied after  $K_v7$  channel blockade with linopirdine (Linop;  $10 \mu\text{M}$ ). Linopirdine on its own did not produce significant changes on spinal reflexes ( $P = 0.069$  for cumulative depolarization and  $P = 0.075$  for spike wind-up; paired  $t$  test). No depressant effects of celecoxib were observed either in cumulative depolarization ( $P = 0.47$ ; paired  $t$  test) or spike wind-up ( $P = 0.81$ ; paired  $t$  test). +++ Significant differences ( $P < 0.001$ ) between predrug and XE-991 values.

$2.68 \pm 0.06 \text{ mm}$  ( $P < 0.001$ ; Wilcoxon test,  $n = 58$ ), and mechanical withdrawal thresholds assayed with von Frey filaments were reduced from a median force of  $1 \text{ g}$  to a median force of  $0.04 \text{ g}$  ( $P < 0.001$ ; Wilcoxon test,  $n = 58$ ).

After spinal cord extraction, spinal reflexes elicited by dorsal root stimulation were recorded from the ventral root. Repetitive dorsal root stimulation at C-fiber intensity induced a progressive potentiation of the responses recorded from the ventral root that manifested as a cumulative depolarization associated with spike wind-up (Fig. 1A). Integrated area of cumulative depolarization and spike wind-up were analyzed to assess the drug effects on nociceptive transmission. The effects of celecoxib at three different concentrations ( $1$ ,  $3$ , and  $10 \mu\text{M}$ ;  $n = 10$ ) were compared with those of the solvent DMSO applied with the same protocol in a separate series of experiments ( $n = 5$ ). A two-way analysis of variance detected significant depressions produced by celecoxib on the cumulative depolarization ( $F[1,13] = 13.34$ ;  $P = 0.003$ ) and the associated spike wind-up ( $F[1,13] = 8.53$ ;  $P = 0.012$ ). The effects of celecoxib were concentration-dependent, and compared with predrug values, the effects on both cumulative depolarization and spike wind-up were statistically significant at concentrations  $\geq 3 \mu\text{M}$  (Fig. 1, C–E).

Dorsal root stimulation caused, in addition, the activation of thick myelinated fibers of muscle origin producing an MSR recorded from the ventral root as a short latency peak (Fig. 1B). Celecoxib did not have significant effects on the MSR ( $F[1,10] = 0.86$ ,  $P = 0.38$ ).

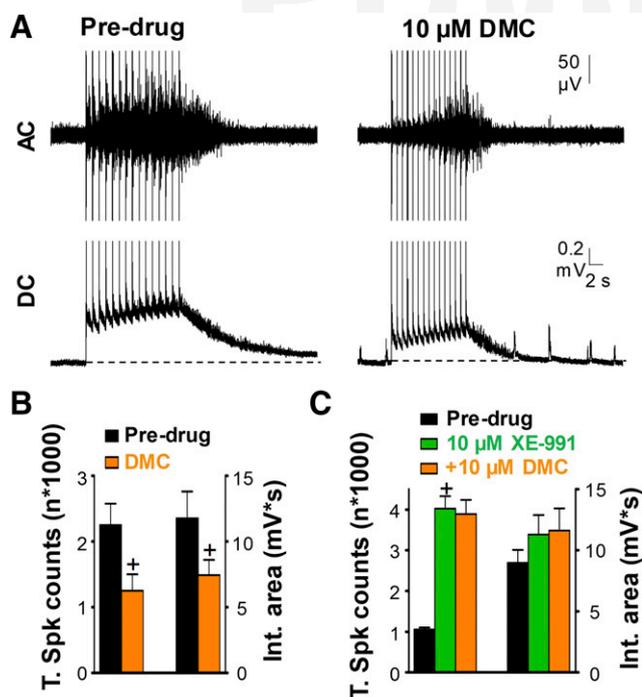
To evaluate the contribution of COX block on celecoxib's actions, we used the irreversible COX blocker indomethacin (Rome and Lands, 1975). To this end, indomethacin was applied at  $10 \mu\text{M}$  during 1 hour, and then  $10 \mu\text{M}$  celecoxib was coapplied during 30 minutes. Indomethacin alone showed no significant effects in cumulative depolarization or spike

wind-up (Fig. 2). However, further application of celecoxib produced a significant depression of both parameters compared with values obtained under indomethacin (Fig. 2).

**Implication of  $K_v7$  Channels in the Actions of Celecoxib on Spinal Reflexes.** The previous results suggest that depressant effects of celecoxib on spinal nociceptive reflexes are mediated by a COX-independent mechanism. To test the possible contribution of  $K_v7$  channels, we studied the effect of  $K_v7$  channel blockade on the depressant action of celecoxib. Superfusion of the  $K_v7$  channel blocker XE-991 at  $10 \mu\text{M}$  on its own produced a strong increase in spike wind-up, but not on the underlying cumulative depolarization ( $n = 7$ ; Fig. 3, A and B). The depressant effects of celecoxib were fully abolished after  $K_v7$  blockade with XE-991 (Fig. 3, A and B). Similar results were obtained using linopirdine, another blocker of  $K_v7$  channels ( $n = 4$ ; Fig. 3C).

To further confirm the implication of  $K_v7$  channels on celecoxib effects, we superfused the cord with DMC, an analog of celecoxib devoid of activity on COX enzymes. DMC applied at  $10 \mu\text{M}$  strongly depressed nociceptive transmission ( $n = 5$ , Fig. 4, A and B). As observed with celecoxib, previous block of  $K_v7$  channels prevented all depressant actions of DMC ( $n = 3$ , Fig. 4C).

**Effects of Celecoxib on the Responses of Dorsal Horn Neurones.** A first series of experiments was conducted to address the potential effects of celecoxib on the spinal sensory neurones that contribute to the initial steps of sensorimotor processing. Extracellular recordings with MEAs were obtained from deep dorsal horn neurones using a hemisectioned spinal cord preparation that allowed us to gain access directly to the gray matter. Twenty-four neurones were successfully isolated from 18 hemisectioned cords. Recording sites recovered after DiI labeling, and histologic processing showed that electrode tracks were located in lamina IV to VI, between  $223$  and  $436 \mu\text{m}$  from the medial side (Fig. 5A).



**Fig. 4.** Actions of the structural analog DMC on nociceptive reflexes. (A) Recordings illustrate the effects of 10  $\mu\text{M}$  DMC on cumulative depolarization ( $P = 0.014$ ; paired  $t$  test) and spike wind-up ( $P = 0.034$ ; paired  $t$  test), showing the same pattern of action than celecoxib. (B) Pooled data from five experiments. (C) Graph summarizing the results obtained in the three experiments that received DMC after  $K_v7$  channels blockade with XE-991. XE-991 application increased spike wind-up ( $P = 0.014$ ; paired  $t$  test) and showed no effect on cumulative depolarization ( $P = 0.15$ ; paired  $t$  test). Further application of DMC was devoid of effect.  $\dagger$  Statistically significant differences ( $P < 0.05$ ) from predrug values as obtained using paired  $t$  test.

Celecoxib was applied at 3 and 10  $\mu\text{M}$  to 13 neurones. Repetitive dorsal root stimulation elicited wind-up responses in three neurones, whereas another eight neurones were able to maintain spike firing during the train of stimuli and the remaining two neurones showed a spike wind-down. In nine of them, action potential firing in response to C-fiber stimulation was depressed by  $\geq 20\%$  (Fig. 5, B and D). Pooling together data from all neurones we obtained statistically significant differences at both concentrations. The four neurones insensitive to celecoxib showed a pattern of maintained firing in response to repetitive dorsal root stimulation.

Occlusion experiments with XE-991 were performed to explore the involvement of  $K_v7$  channels in the effects of celecoxib. Responses to repetitive stimulation in predrug conditions for the five neurones tested included wind-up responses (two), neurones maintaining spike firing (two), and one neurone showing spike wind-down. A 60-minute application of XE-991 at 10  $\mu\text{M}$  produced a mild but significant change in neuronal responses to repetitive stimuli ( $n = 5$ ). After the superfusion of XE-991, applications of celecoxib at 3 and 10  $\mu\text{M}$  were completely devoid of effects in the five neurones tested (Fig. 5, C and E).

In an additional set of experiments, we wanted to explore the effects of celecoxib on neurones more specifically related to nociceptive processing. Using a slice containing the laminae from I to V of the dorsal horn, we obtained MEAs recordings of the activity of superficial dorsal horn neurones in response to repetitive dorsal root stimulation at C-fiber intensity. Twenty-one

superficial dorsal horn neurones were isolated from eight spinal cord slices. Electrode tips were situated at 20–60  $\mu\text{m}$  from the dorsal border of the spinal cord, allowing all the sensors to be located within laminae I–III (see Fig. 6A).

Celecoxib applied at 10  $\mu\text{M}$  depressed the responses to dorsal root stimulation in 10 out of 12 neurones tested (Fig. 6, B and D). Neurones inhibited by celecoxib showed wind-up (two), wind-down (three), or maintained (five) responses to repetitive stimulation. Two more neurones insensitive to celecoxib had wind-up responses.

Occlusion experiments with XE-991 were made in nine additional neurones showing wind-up (five), wind-down (two), or maintained firing (two). XE-991, applied at 10  $\mu\text{M}$  during 60 minutes, produced slightly increased responses in seven neurones, but this trend did not reach statistical significance. The posterior coapplication of 10  $\mu\text{M}$  celecoxib was devoid of effects in the nine neurones tested (Fig. 6, C and E).

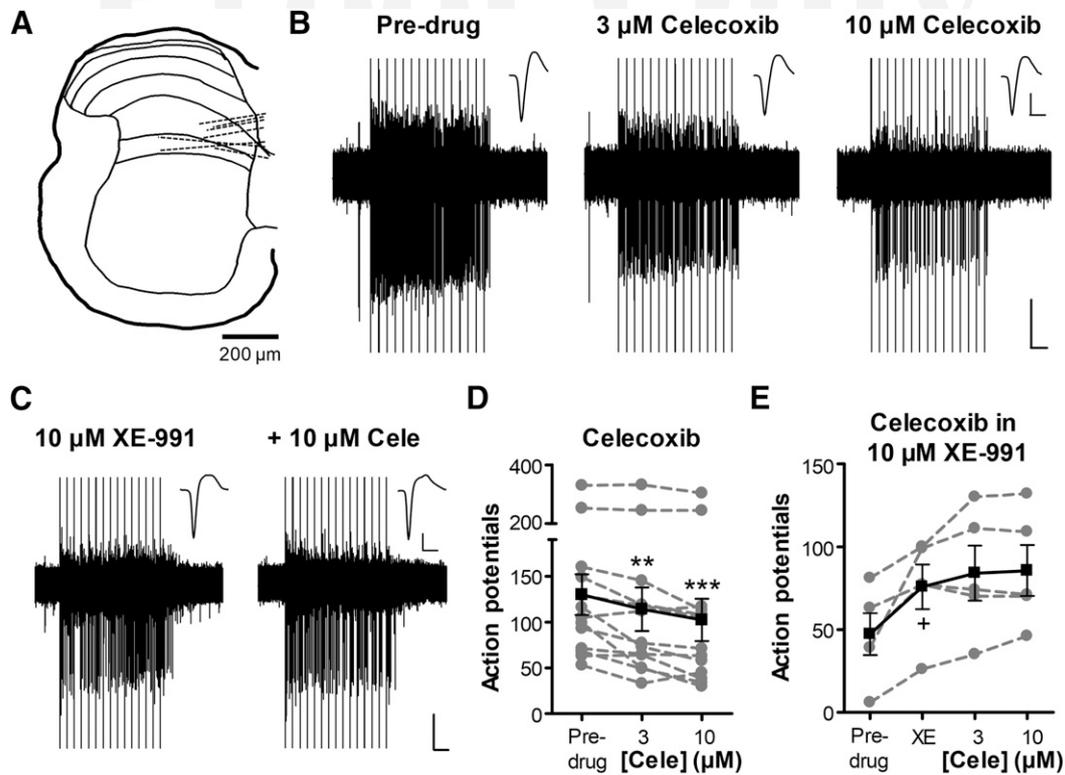
## Discussion

Applications of celecoxib to the in vitro mouse spinal cord produced a concentration-dependent depression of nociceptive reflexes, manifested as a reduction of action potential wind-up and the underlying cumulative depolarization, a model widely used to study nociceptive processing in the spinal cord (Herrero et al., 2000). On the other hand, the monosynaptic reflex, a purely non-nociceptive response, was unaffected by celecoxib. This profile of action on spinal reflexes is very similar to that of other analgesics, including morphine (Mazo et al., 2015). In addition, celecoxib reduced the firing of dorsal horn neurones in response to C-fiber stimulation, indicating a specific action of the compound on sensory neurones. These results are in line with previous reports showing that celecoxib applied by intrathecal route reduces nociceptive behaviors evoked by formalin injection in the hind paws (Nishiyama, 2006; Lee and Seo, 2008).

The present results show significant depressant effects of celecoxib at concentrations  $\geq 3 \mu\text{M}$ . These are in range with plasmatic levels of celecoxib achieved in humans after treatment (100–400 mg, 1–4  $\mu\text{M}$ ) (Davies et al., 2000), which suggests that the spinal actions of celecoxib may be relevant to its analgesic effects under clinical conditions.

In this work, carrageenan-inflamed mice were used in an attempt to maximize COX activity. Both COX isozymes are constitutively present in the spinal cord, and their expression appears to up-regulate under neuropathic and inflammatory processes (Hay and de Belleruche, 1997; Zhu and Eisenach, 2003). However, some reports suggest that spinal COX activity is not relevant to acute pain transmission nor to the maintenance of hyperalgesia in inflammatory models (Yamamoto and Nozaki-Taguchi, 1996; Dirig et al., 1998; Vardeh et al., 2009). This view is reinforced by the lack of effect of indomethacin on spinal reflexes and the large, likely unspecific concentrations of other NSAIDs required to cause depression (Lopez-Garcia and Laird, 1998; Lizarraga et al., 2006).

Our carrageenan-inflamed mice showed signs of edema and allodynia. Previous reports using this in vitro model have shown larger spinal reflexes in treated rats than in controls (Hedo et al., 1999). In our study, the application of indomethacin at 10  $\mu\text{M}$ , a concentration above its therapeutic range and  $\text{IC}_{50}$  values for COX inhibition (Kurumbail et al., 1996;



**Fig. 5.** Effects of celecoxib on the activity of deep dorsal horn neurones. (A) Drawing of a transverse section of the hemisected spinal cord (dorsal side up) with a reconstruction of its borders and laminar organization. The approximate location of the multielectrode shanks used for recording were recovered after DiI labeling. Dotted lines indicate the trajectory of the electrodes inside the spinal cord recovered from the seven experiments in which labeling with DiI was obtained. (B) Original recordings showing celecoxib (3 and 10  $\mu\text{M}$ ) effects on action potential firing in response to repetitive high-intensity stimulation of the dorsal root as obtained from a deep dorsal horn neurone. (C) Illustration of the lack of effect of celecoxib after  $K_v7$  channel blockade with XE-991. Celecoxib at 3 and 10  $\mu\text{M}$  was coapplied with 10  $\mu\text{M}$  XE-991, but only the higher concentration is shown. Insets show the average of all action potentials in each condition. Calibration bars for all recordings: 0.2 mV and 2 seconds (0.2 mV and 1 millisecond for insets). (D) Graph showing the effects of celecoxib on action potential firing in response to repetitive dorsal root stimulation in the 13 neurones tested. Celecoxib had depressant effects on neuronal firing ( $F[2,24] = 15.28$ ;  $P < 0.001$ ; one-way ANOVA). The vertical axis was broken to improve visualization. (E) Graph summarizing the effects of celecoxib at 3 and 10  $\mu\text{M}$  after  $K_v7$  channel blockade with XE-991 in the five neurones tested. XE-991 increased the firing of deep dorsal horn neurones ( $P = 0.026$ ; paired  $t$  test). In the presence of XE-991, the coapplication of celecoxib was devoid of effect ( $F[2,8] = 1.58$ ;  $P = 0.26$ ; one-way ANOVA). For both graphs, the values for individual neurones are shown in gray; mean  $\pm$  S.E.M. values for all neurones included in the graph are indicated in black. Asterisks (\*\* $P < 0.01$  and \*\*\* $P < 0.001$ ) and plus signs (+ $P < 0.05$ ) indicate statistically significant differences from predrug values as obtained using the Bonferroni post hoc test after one-way ANOVA or paired  $t$  test, respectively.

Kalgutkar et al., 2000; Lucas, 2016), was devoid of effect on spinal reflexes, whereas subsequent coapplication of celecoxib produced clear depressant actions. These observations suggest a minor role for COX blockade in the actions of celecoxib on spinal transmission and a likely contribution of other unspecific targets for this compound.

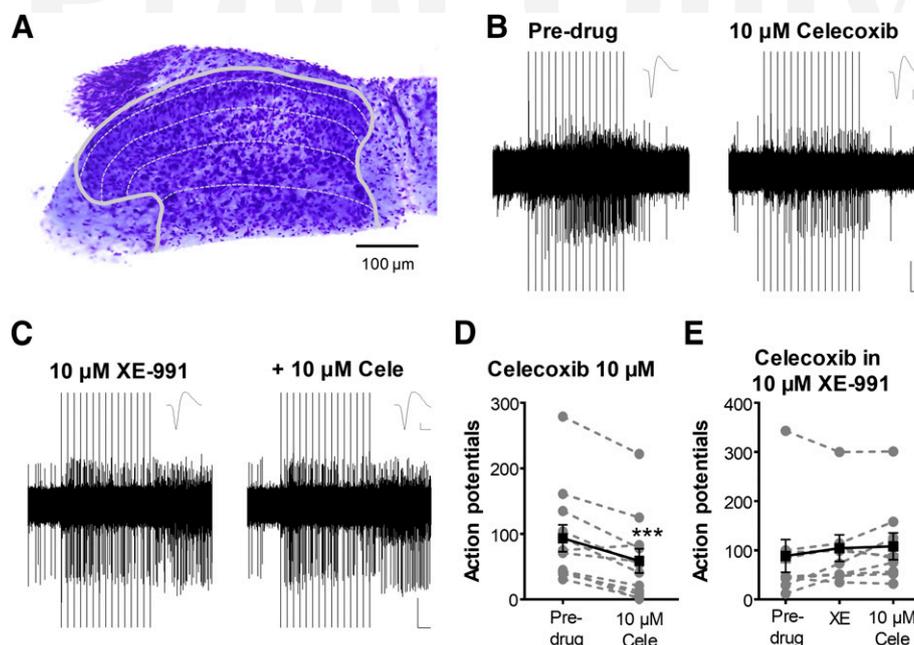
Celecoxib applied at concentrations similar to those used in the present experiments has been shown to exert direct actions on heterologous expressed  $K_v7$  channels and on native M-currents expressed in dorsal root ganglia neurones (Brueggemann et al., 2009; Du et al., 2011; Mi et al., 2013). A recent report has shown that  $K_v7$  subunits 2, 3, and 5 are present in the spinal cord and are expressed in both inhibitory and excitatory dorsal horn neurones (Haring et al., 2018). Interestingly, they found that the  $K_v7.5$  subunit is expressed in two populations of glutamatergic neurones (glut 11 and 12), which became activated by noxious thermal stimulation. In this line, other work employing a similar approach (Sathya-murthy et al., 2018) also reported the expression of  $K_v7.5$  subunits in a population of excitatory neurones (DE-7) that express the Fos gene after formalin application.

Celecoxib can interact with several homo- and heteromeric conformations of  $K_v7$  channels showing  $EC_{50}$  values between

2 and 5  $\mu\text{M}$ . Celecoxib shifts the activation curve of  $K_v7$ -mediated currents to the left and produces an approximately 2-fold increase in conductance (Du et al., 2011). In previous studies, we showed that retigabine, the standard  $K_v7$  channel opener, has depressant actions on spinal reflexes comparable to those obtained here for celecoxib (Rivera-Arconada et al., 2004; Vicente-Baz et al., 2016). Like celecoxib, retigabine has similar affinity for different  $K_v7$  channel subunits and  $EC_{50}$  values in the low micromolar range (Tatulian et al., 2001; Wickenden et al., 2001).

The major proof obtained here in favor of the implication of  $K_v7$  channels in mediating the spinal effects of celecoxib comes from the experiments with  $K_v7$  channel blockers. Under the present conditions, XE-991 showed clear potentiating actions on the firing of motor neurones while the effects on dorsal horn neurones were smaller. A minor effect of XE-991 on dorsal horn neurones has been observed in intracellular recordings obtained in rats (Rivera-Arconada and Lopez-Garcia, 2005). The lack of effect of XE-991 on cumulative depolarization may be explained by the little impact that this compound has on dorsal horn neurones and, consequently, on their synaptic input to motor neurones.

The concentration of XE-991 used here was sufficient to produce complete blockade of the effects of the specific



**Fig. 6.** Effects of celecoxib on the activity of superficial dorsal horn neurones. (A) Microphotograph showing a transverse section of the spinal cord slice stained with toluidine (dorsal side up) with a reconstruction of its borders and laminar organization. The slice contained both sides of the spinal cord, but only one side is shown in the image for clarity. (B) Original recordings showing an example of the effects of 10  $\mu\text{M}$  celecoxib on action potential firing. (C) Illustration of the lack of effect of celecoxib after  $\text{K}_v7$  channel blockade with XE-991. Insets show the average of all action potentials in each condition. Calibration bars for all recordings: 0.3 mV and 2 seconds (0.2 mV and 1 millisecond for insets). (D) Graph showing the effects of celecoxib on action potential firing in response to repetitive dorsal root stimulation in the 12 neurones tested. Celecoxib had depressant effects on neuronal firing ( $P < 0.001$ ; paired  $t$  test). (E) Graph summarizing the effects of celecoxib at 10  $\mu\text{M}$  after  $\text{K}_v7$  channel blockade with XE-991 in the nine neurones tested. XE-991 had no effect, but prevented those of celecoxib. For both graphs, values for individual neurones are shown in gray; mean  $\pm$  S.E.M. values for all neurones included in graph are indicated in black. Asterisks (\*\*\*)  $P < 0.001$  indicate statistically significant differences from predrug values as obtained using a paired  $t$  test.

$\text{K}_v7$  opens *N*-(2,4,6-trimethylphenyl)-bicyclo[2.2.1]heptane-2-carboxamide (ML213) and *N*-(2-chloro-5-pyrimidinyl)-3,4-difluorobenzamide (ICA-069673) in previous experiments (Vicente-Baz et al., 2016). We have also proof to our satisfaction that XE-991 does not prevent the effects of 1  $\mu\text{M}$  diazepam, an allosteric modulator of GABA-A receptors under identical experimental conditions (not shown). Thus, we believe that the effects of XE-991 should be considered effective and selective for  $\text{K}_v7$  channels. Under these conditions of specific  $\text{K}_v7$  channel blockade, celecoxib had no effect on spinal reflexes. Also relevant is the finding that XE-991 prevented the depressant effects of celecoxib on the responses of dorsal horn neurones to C-fiber activation. This shows that early sensory processing of nociceptive information is depressed by celecoxib using a mechanism that likely involves  $\text{K}_v7$  channels as well.

Further evidence supporting the implication of  $\text{K}_v7$  channels on celecoxib depression of spinal nociceptive transmission was obtained in experiments with DMC, a structural analog of celecoxib that lacks COX blocking activity (Kardosh et al., 2005). This compound produced depressant effects on spinal reflexes similar to those of celecoxib, that were also abolished by XE-991 application.

All these observations support a role for  $\text{K}_v7$  channels on celecoxib action although a minor contribution of other targets might be possible. Celecoxib can interact with other targets like sodium and calcium channels which may also contribute to its observed effects (Park et al., 2007; Brueggemann et al., 2009). Although experiments with XE-991 may suggest direct actions on  $\text{K}_v7$  channels,  $\text{K}_v7$  channel blockade may depolarize

resting potential originating an inactivation of sodium and calcium channels, also preventing celecoxib effects mediated by these other channels. We have shown previously that spinal neurones can be depolarized by the application of 10  $\mu\text{M}$  XE-991 (Rivera-Arconada and Lopez-Garcia, 2005). Depolarizations ranged from 1 to 5 mV, with higher values found in motor neurones, which also showed more negative potentials. These small depolarizations are unlikely to substantially affect high-threshold calcium channels or sodium channels to avoid subsequent actions of celecoxib. However, further studies will be necessary to definitively rule out this possibility.

In summary, the present results prove the concept that  $\text{K}_v7$  channels are relevant potential mediators of the antinociceptive actions of celecoxib acting on the spinal cord. Although the results have to be interpreted cautiously due to the limitations of the present experimental approach, it is clear that celecoxib produces depression of the responses of sensory neurones and spinal nociceptive reflexes activating nonspecific targets. Although several nonspecific targets have been identified, the present results are consistent with an involvement of  $\text{K}_v7$  channels in these actions of celecoxib. In conclusion, we suggest that celecoxib may combine its main peripheral actions as a blocker of COX enzymes, with activity at  $\text{K}_v7$  channels located in spinal nociceptive circuits to achieve analgesia.

#### Authorship Contributions

Participated in research design: Lopez-Garcia, Rivera-Arconada.  
 Conducted experiments: Vicente-Baz.  
 Performed data analysis: Vicente-Baz, Rivera-Arconada.

Wrote or contributed to the writing of the manuscript: Vicente-Baz, Lopez-Garcia, Rivera-Arconada.

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